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PROGRAM
AND ABSTRACTS
OF PAPERS
CITRUS
RESEARCH
CONFERENCE

FRUIT AND VEGETABLE
CHEMISTRY LABORATORY
263 SOUTH CHESTER AVENUE
PASADENA, CALIFORNIA 91106
December 2, 1969

Western Utilization Research and Development Division
Agricultural Research Service
UNITED STATES DEPARTMENT OF AGRICULTURE



FOREWORD

This Citrus Research Conference is being held to bring to members of the citrus and allied industries in Southern California and Arizona the latest results of research on the chemistry, pharmacology, and technology of citrus fruits and their products carried on by the Utilization Research and Development Divisions of the Agricultural Research Service, U. S. Department of Agriculture. The following are participating in this year's conference.

- Western Utilization Research and Development Division:
 - Western Regional Research Laboratory (Division headquarters), 800 Buchanan Street, Albany, Calif. 94710
 - Fruit and Vegetable Chemistry Laboratory, 263 South Chester Avenue, Pasadena, Calif. 91106
- Southern Utilization Research and Development Division:
 - U. S. Fruit and Vegetable Products Laboratory, 600 Avenue S, N.W., Winter Haven, Florida 33882
 - U.S. Food Crops Utilization Research Laboratory
 P.O. Box 388, Weslaco, Texas 78596

PROGRAM

CITRUS RESEARCH CONFERENCE

Tuesday, December 2, 1969

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SYNTHESIS, STRUCTURE AND TASTE OF SOME NEW FLAVONOID GLYCOSIDES

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Pasadena, California

In continuation of our studies on the relation of taste to the structure of flavonoid glucosides and neohesperidosides, we have prepared several new flavanone and dihydrochalcone glycosides. Of the various sweet dihydrochalcones known at present, neohesperidin dihydrochalcone appears to be the most promising from a practical point of view since it is quite water soluble and has a very high level of sweetness. The Bring of neohesperidin dihydrochalcone contains 3-hydroxy and 4-methoxy substituents (I). It appeared to us that the addition of a 5-hydroxy

substituent (to give II) might reasonably be expected to enhance the sweetness and solubility of this compound. In fact, the expectation was only partially realized. Problems encountered in the synthesis of this and some other new dihydrochalcones and flavanones will be reviewed and a discussion of their taste properties given.

Isolation studies on Seville oranges (<u>Citrus aurantium</u>) have shown the presence of the neohesperidosyl derivatives neoeriocitrin and neodiosmin. The identification and possible taxonomic significance of these compounds will be discussed.

COMPOSITION AND INHERITANCE OF FLAVANONES IN CITRUS FRUIT

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Weslaco, Texas

A thin-layer chromatographic survey was made of flavanones present in 41 citrus varieties representing 18 recognized species of citrus. In addition, 49 hybrids of 18 different crosses were also surveyed. An analysis of the data has shown that a qualitative and quantitative consistency of flavanone composition, with minor variations, is characteristic of individual citrus species and crosses. Rules governing the inheritance of citrus flavanones were deduced from the composition of known hybrids. These rules coupled with compositional data were used to evaluate the probable taxonomic relationships of various citrus varieties and species.

The rules governing the inheritance of the bitter flavanones may be of value to the citrus breeder. A knowledge of the flavanone composition characteristic of a particular citrus species or cross may be useful to the processing industry for determining the varietal composition of citrus products.

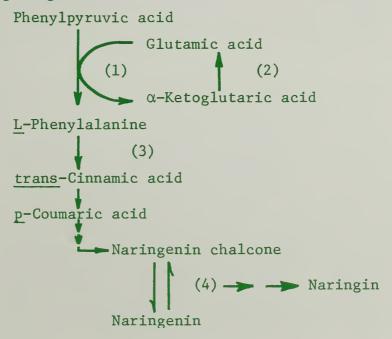
ENZYMES INVOLVED IN BIOSYNTHESIS OF NARINGIN IN GRAPEFRUIT

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Fruit and Vegetable Chemistry Laboratory
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Naringin is the principal flavonoid constituent of grapefruit. Because of its extreme bitterness, naringin when present in excessive amounts is considered to be a prime factor in limiting the acceptance of grapefruit. The presence of naringin in grapefruit has been known for many years, and its physical and chemical characteristics have been well established, but the knowledge of the biosynthesis of naringin in grapefruit is very limited. Although a general working hypothesis for the pathway of naringin biosynthesis can be deduced from radiotracer studies of phenolic biosynthesis in other plants and from knowledge of the phenolic constituents found in grapefruit, most of these steps have not been demonstrated in grapefruit. Even more important, the enzymes catalyzing each of these steps in the deduced pathway have not been demonstrated in grapefruit.

Because our main objective is to learn what controls naringin production and accumulation in the fruit, studies have been undertaken to determine what enzymes are involved in the biosynthetic pathway, to measure the activities of the enzymes and to correlate their activities with the rate of naringin accumulation.

During the past two years of our investigation, we have identified four enzymes which are related to naringin biosynthesis in grapefruit as seen in the following diagram:



The first enzyme identified was phenylalanine: α -ketoglutarate aminotransferase (1). It catalyzes the production of L-phenylalanine from phenyl-pyruvic acid. In this reaction glutamic acid serves as the amino group donor and is itself converted to α -ketoglutaric acid. The second enzyme identified was glutamate dehydrogenase (2). It catalyzes the regeneration of glutamic acid from α -ketoglutaric acid in the presence of NADH and NH3. During development and growth, the fruit possessed relatively high glutamate dehydrogenase activity.

The third enzyme identified was L-phenylalanine ammonia-lyase (PAL) (3). This enzyme catalyzes the deamination of L-phenylalanine and yields trans-cinnamic acid as a product. We found that young fruit possessed very high PAL activity and that the PAL activity decreased sharply as the fruit grew in size. In addition, a direct relationship was observed between PAL activity and the rate of naringenin glycoside accumulation in the developing fruit. The fruit tissues had potential PAL activity sufficient to account for the accumulated naringenin glycosides and still allow for turnover and synthesis of them and other cinnamate derived phenols. These results suggested that PAL is an element in the system that regulates naringin synthesis in grapefruit.

The fourth enzyme identified was chalcone-flavanone isomerase (4). This enzyme catalyzes the interconversion of chalcones and flavanones. Shimokoriyama (1957) previously reported evidence of chalcone-flavanone isomerase activity in the peel of several other citrus fruits.

Thus, four enzymes essential for naringin biosynthesis have been shown to be present in grapefruit. Also, PAL activity has been shown to correlate nicely with the rate of naringenin glycoside accumulation. These results strongly indicate that fruit tissues have the capability of synthesizing naringin starting at least from the phenylpyruvate stage.

ANALYTICAL AND BIOCHEMICAL ASPECTS OF THE LIMONIN BITTERNESS PROBLEM

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Studies underway on the biochemistry of limonoids in citrus fruits required the development of a specific and sensitive quantitative method for determining limonin in all types of citrus tissues and juices. Such a method has been developed by combining the ability of thin-layer chromatography (TLC) to separate limonin from other citrus constituents (Maier and Dreyer, J. Food Sci. 30:874-75, 1965) and the selectivity and sensitivity of Ehrlich's reagent for limonoids (Dreyer, J. Org. Chem. 30:749-51, 1965) with the general extraction procedures of Wilson and Crutchfield (J. Agr. Food Chem. 16:118-24, 1968). In this TLC assay limonin, once it is formed in citrus juices or acidified tissue suspensions, is removed by two extractions with chloroform; the chloroform extracts are combined and evaporated; and the residue is dissolved in a measured volume of acetonitrile. The acetonitrile solution is quantitatively spotted on a silica gel thin-layer chromatogram along with authentic limonin; the chromatogram is developed in benzene:ethanol:water:acetic acid (200:47:15:1, upper phase), dried, sprayed with Ehrlich's reagent, and treated with gaseous HCl. Readout is by visual or spectrodensitometric comparison of the unknown and known limonin spot densities.

Reproducibility of the method was tested with samples of lemon, orange, and grapefruit juices and their concentrates; it was routinely \pm 5 percent and better. Recoveries of limonin added to the above samples were quantitative over the range 0.5 to 500 p.p.m. On a routine basis it was possible to determine limonin contents as low as 0.5 p.p.m. with 20-gram sample of juice. Lower limonin concentrations have been determined by using larger samples.

In addition to its use in determining the limonin content of citrus juices, the TLC assay can also be used to determine indirectly the limonoate A-ring lactone content of fruit tissues by acid conversion of this nonbitter precursor to limonin. With this approach, it was found that in a yellow-ripe lemon the various tissues of the fruit contained the following percentages of total potential limonin: peel 74.0 percent, section membranes 20.2 percent, juice sac walls 3.3 percent, and cheesecloth filtered juice 2.5 percent. This juice, prepared directly from the carefully separated juice sacs by pressing on cheesecloth with suction removal of the juice, contained 8 p.p.m. limonin. Thus, even when the juice is prepared in a very gentle manner a significant amount of limonoate A-ring lactone enters the juice and is converted into limonin. If all of the limonoate A-ring lactone contained in the juice sac walls had entered the juice (a possibility in commercial juice preparation) the limonin content

would have been 18 p.p.m., and the juice would have been extremely bitter. If, in addition, small percentages of the limonoate A-ring lactone of the section membranes and peel had entered the juice its limonin content would have been considerably higher. These figures point out the extreme importance of gentle juice extraction procedures and short contact time between juice and pulp to minimize limonin bitterness in the juice.

Other data dealing with postharvest changes in limonoate A-ring lactone content of citrus fruits will be discussed, along with recent results on the properties of the enzyme limonin D-ring-lactone hydrolase.

PATTERN OF NOOTKATONE FORMATION IN RIPENING DESERT GRAPEFRUIT

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Previously we reported on the pattern of carotenoid formation in desert grapefruit. We had observed that in ripening fruit the development of the ripe color begins prior to the decrease in chlorophyll (green color). During this period of active carotenoid formation the colorless C-40 precursor phytoene does not accumulate; the ¹⁴C mevalonic acid is readily incorporated into the colored carotenoids. However, as the fruit ripens further to the yellow stage the colorless C-40 precursor phytoene accumulates, and a decrease in the net synthesis of total carotenoids is observed. The ¹⁴C mevalonic acid is no longer incorporated into the carotenoids. This suggests a blockage at the primary C-40 step (phytoene) in the sequential pathway leading to the formation of the carotenoids. Thus it was of interest to investigate the effect of this observed blockage on the sesquiterpenoid nootkatone, the important flavoring constituent of grapefruit which shares common biosynthetic intermediates with the carotenoids.

Nootkatone was examined at various stages of maturity of the desert grapefruit. In the ripening grapefruit the formation of nootkatone appears subsequent to the accumulation of phytoene; nootkatone is not present in detectable amounts in the green fruit. It is essentially with the disappearance of chlorophyll and of the yellow color that nootkatone is first detected. The buildup of nootkatone continues until full maturity. The pattern of formation of nootkatone in the ripening desert grapefruit will be described in detail.

NEW APPROACHES TO REDUCING ACIDITY AND STABILIZING CLOUD IN CITRUS JUICES

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Our research on enzyme reactions in citrus has provided leads toward solving two important problems in citrus processing. By defining the substrate requirements for the enzymic synthesis and degradation of citric acid, we now understand how acidity can be controlled in citrus fruit. We are using this information to develop a method that would significantly reduce the total acidity of orange and grapefruit juice. By separating the role of pectin in "cloud" stabilization from its role as substrate for pectinesterase, we have been able to devise an approach toward stabilizing orange and grapefruit juices without heating.

STUDIES ON THE STABILITY OF LEMON OIL I. THE HYDROCARBON FRACTIONS OF RAW, COLD-PRESSED CALIFORNIA LEMON OIL

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The development of off-flavors and -odors in commercial lemon oil has been attributed to the lability of their terpene hydrocarbons. Ikeda et al. (Food Technol. 15:379, 1961) presented evidence indicating that p-cymene was formed from γ -terpinene when lemon oil samples were stored in inadequately stoppered bottles. However, additional chemical changes may occur under different storage conditions and after addition to food and other products as a flavoring ingredient. Improved gas-liquid chromatography (GLC) techniques have permitted a more detailed examination of constituents and a systematic study of chemical changes involved in lemon oil deterioration.

Raw, cold-pressed lemon oil was molecularly distilled to remove high boiling waxes and nonvolatile material. The distillate and more volatile material, caught in a low temperature trap, were recombined and chromatographed on an alumina column. The hydrocarbons were eluted preferentially with pentane. Separation of the hydrocarbons from the oxygenated compounds facilitated resolution of individual constituents in the gas chromatograph. Twelve apparent monoterpene hydrocarbons were separated on a 5 percent Apiezon L GLC column at 78°C. The resolved constituents were identified tentatively as α -thujene, α -pinene, camphene, sabinene, myrcene, β -pinene, α -phellandrene, α -terpinene, p-cymene, limonene, γ -terpinene and p- α -dimethylstyrene.

A comparison of the hydrocarbon fractions obtained from authentic Coastal and Desert lemon oil samples confirmed previous reports of significant differences in total amount and relative proportions of their monoterpene hydrocarbons. The ratios of limonene: β -pinene found for Coastal and Desert oil were 3.8:1 and 13.5:1, respectively. Coastal oil contained 15.9 percent β -pinene, about three times as much as in Desert oil. The Coastal oil also contained larger amounts of all other monoterpenes except myrcene.

The higher boiling hydrocarbons, which composed only slightly more than 1 percent of the oil samples, were separated on a 5 percent Apiezon L GLC column at 169°C. Twenty additional hydrocarbons were resolved including apparent dodecane, tetradecane, pentadecane, bergamotene, α -carophyllene, β -bisabolene, valencene and 12 unidentified constituents. Coastal oil contained slightly larger amounts of higher boiling compounds although the relative proportions of the constituents in the two oils were similar.

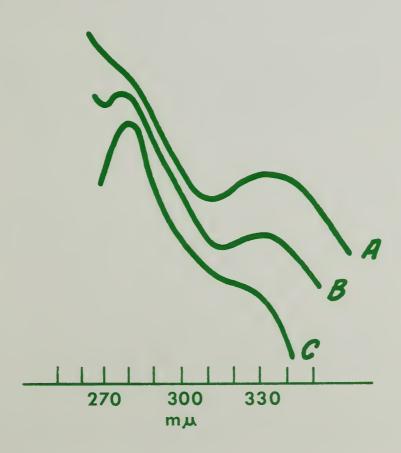
Storage of Desert lemon oil in a sealed, flint glass bottle with antioxidant under ambient daylight conditions caused rapid bleaching, significant flavor change, and marked alterations among the monoterpene hydrocarbons. The p-cymene: γ -terpinene ratio exhibited a 25-fold change during a 6-month storage period. During the same period, all of the α -phellandrene, α -terpinene and most of the apparent p- α -dimethylstyrene were lost. Similar but less dramatic changes occurred among several of the higher boiling hydrocarbons, including the sesquiterpene β -carophyllene and two unidentified constituents. These changes did not occur with identical samples stored in either amber or Pyrex glass bottles. Light irradiation is an important factor in the decomposition of monoterpenes and the development of off-flavors and -odors in whole lemon oil.

CHANGES IN ULTRAVIOLET SPECTRAL PROPERTIES OF LEMON JUICE UNDER ADVERSE STORAGE CONDITIONS

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During analysis of samples of fresh and commercial lemon juice it was observed that the wavelength of a peak in the ultraviolet spectra of the juice would range between 323 and 335 mµ. These shifts in the peak and general shape of the spectra appeared to depend on juice storage time and other conditions. Some of the factors influencing these spectral changes will be discussed.

A typical spectrum of a fresh lemon juice sample is shown in (A) below. The wavelength of the maximum falls between 327and 335 m μ , depending upon the sample. In the 275-m μ region there is an inflection point or shoulder. As the juice deteriorates, the 330-m μ peak gradually shifts to shorter wavelengths (B) and the minimum eventually disappears; the peak at 275 m μ becomes quite prominent. The spectra of a badly deteriorated sample of lemon juice is represented by (C).



A useful parameter relating to the freshness or state of deterioration of the lemon juice sample may be calculated from the slope of a line from the maximum to minimum absorbance (or slope at the inflection point) divided by the absorbance at the maximum or inflection point. Values of the slope ranged between 0.0060 to 0.0040 for fresh lemon juice.

$$S = \frac{E_{\text{max}} - E_{\text{min}}}{E_{\text{max}} (\lambda_{\text{max}} - \lambda_{\text{min}})}$$

$$S = \text{normalized slope}$$

$$\lambda_{\text{max}} = \text{wavelength at peak } (330 \pm 5 \text{ m}\mu)$$

$$\lambda_{\text{min}} = \text{wavelength at minimum } (310 \text{ to } 315 \text{ m}\mu)$$

$$E_{\text{max}} = \text{absorbance at } \lambda_{\text{max}}$$

$$E_{\text{min}} = \text{absorbance at } \lambda_{\text{min}}$$

Most samples of commercial frozen lemon juice concentrate reconstituted and tested in this laboratory had slope values falling between 0.0050 and 0.0030. Some shelf-pack items purchased from local markets had spectral slopes around zero. The changes in the spectral properties due to the presence of air in the juice container and the temperature of storage were determined. As one might expect, both air and high temperature storage have a detrimental effect. It was also observed that there was no large change in the slope of the spectra until considerable ascorbic acid had been oxidized.

As a juice deteriorates and the slope of its spectrum changes, there are some important changes taking place in the flavor and color. Undesirable odors and off-flavors may be detected when the slope drops below 0.0010. When the slope is around zero the juice is noticeably darker than fresh juice. Juice with a negatively sloped spectrum has a definite browned appearance and would not be acceptable to most consumers. The spectra of all lemon juice samples examined thus far follow the same pattern of changing slope and wavelength of the peak with deterioration. Therefore, it might be possible to use the slope as an index of freshness or of the onset of browning. A properly stored juice would maintain a relatively high positive slope for long periods. On the other hand, an improperly stored juice could be detected by a low or negative slope.

THE JOFFEH JAFFA OR OBSERVATIONS ON THE CITRUS INDUSTRY IN ISRAEL

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Israel is a nation searching for a livelihood. It lacks raw materials for manufacture, has a limited water, power, and labor supply, and has the highest labor costs and standard of living of any country in the Middle East. In order to improve the balance of trade the government is pressing hard with special concessions and benefits to businesses to encourage exports. The largest single export business is the citrus industry. Export of fresh fruit earned an annual \$85 million during the past 2 years. Processors utilize packing house rejects or surplus fruit. The Israeli Citrus Marketing Board closely controls fresh product quality and export distribution.

Last season 300,000 tons of fruit (about 30 percent of production) was processed. With most of the Eastern European Market closed to them, the specter of over-production haunts the growers. Hence the interest in having a strong products industry. Nevertheless, in some seasons it has been necessary to purchase concentrate from the United States to meet production commitments. There are sixteen companies in Israel processing citrus. The largest at full capacity handles 800 tons of fruit per day. One or two only process fruit sections. Companies also process other fruits and vegetables, nuts, fish, etc. in order to keep manpower and equipment employed as much of the year as possible. Only relatively small amounts of frozen products have been processed in the past, but these are now showing a definite increase.

When the citrus processing industry was started under Dr. Braverman's able direction, much improvisation was necessary and several very practical locally designed pieces of equipment were used. Only this year, the last Koffler juice presses were replaced. Now we see much modern equipment from England, Switzerland, Germany, and the United States. One plant, for example, has two Centritherm units in its line. The industry draws heavily on research and experience from the United States. It is well served, and increasingly so, by local research institutes - The Volcani Institute, Rehovot; the Food Engineering Department of the Technion, Haifa, where most technical personnel have been trained; Tel Aviv University; the Central Citrus Research Laboratory, Rehovot (formerly the Braverman Laboratory); by groups at the Hebrew University, Jerusalem; and by the newly organized Centre for Industrial Research, Haifa.

The problem baby is the Shamouti orange (given the regional name Jaffa) which is the major fruit available for processing (Navels and

Valencias are grown in limited amounts). Though an excellent fresh fruit (780,000 tons were exported last year), the Shamouti presents many problems to the processor: high acidity, poor color, low flavor, low oil and juice yields, low aldehyde levels in peel oils (and reduced value in folding), and gelation in high brix concentrates. Various aspects of these problems have been and are now under intensive study.

Lemons are grown in small amounts. Thirty-nine thousand tons were shipped last year. The trees suffer from mal secco disease. Three hundred thousand tons of grapefruit were shipped last season. Grapefruit products are very popular locally.

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^{*}Reprints are available at the addresses indicated; patents are available only by purchase at 50¢ a copy from the U. S. Patent Office, Washington, D. C. 20231.

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